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13. ABSTRACT (Maximum 200 Words) Mutational inactivation of the breast cancer susceptibility gene, <i>BRCA1</i> , accounts for a large percentage of hereditary breast cancer. Recently, the highly conserved ring finger domain of <i>BRCA1</i> has been shown to function as an ubiquitin protein ligase or E3 in the ubiquitination of model substrates. Our hypothesis is that <i>BRCA1</i> effects the ubiquitination of proteins that are either negative regulators of DNA repair or are positive regulators of growth proliferation, leading either to their degradation or to an alteration of their activity. A major advancement in the understanding of <i>BRCA1</i> and its putative E3 activity, would be the identification of bonafide <i>BRCA1</i> substrates. To this end, we have generated the reagents to reconstitute <i>BRCA1</i> -dependent ubiquitination <i>in vitro</i> using E1, E2 (UbcH5b), and full-length baculovirus-expressed <i>BRCA1</i> and BARD1. It is expected that only full-length <i>BRCA1</i> and BARD1 proteins will confer physiologically relevant substrate specificity. We are currently testing Estrogen Receptor α , RNA Polymerase II large subunit, and <i>BRCA1</i> -interacting proteins as potential <i>BRCA1</i> /BARD1 substrates. We are also generating stable cell lines which express epitope-tagged <i>BRCA1</i> for the purification of the <i>BRCA1</i> E3 complex from mammalian cells and we are developing an <i>in vivo</i> assay for the identification of potential <i>BRCA1</i> /BARD1 substrates.					
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THE ROLE OF BRCA1-DEPENDENT UBIQUITINATION IN BREAST CANCER

INTRODUCTION

Mutational inactivation of the Breast Cancer Susceptibility Gene, *BRCA1*, accounts for a large percentage of hereditary breast cancer. *BRCA1* has been shown to function in a number of different cellular processes, yet it is still unclear how *BRCA1* biochemically mediates its cellular function as a tumor suppressor protein. Recently, the highly conserved RING finger domain of *BRCA1* has been implicated in the ubiquitination of proteins and is thought to function as a RING finger-type ubiquitin protein ligase or E3 enzyme (1-9). However, how the putative E3 activity of *BRCA1* may mediate the functions of *BRCA1* in the cell remains unclear. Our hypothesis is that *BRCA1* mediates its biological function by targeting proteins for ubiquitination. We propose that *BRCA1* mediates the ubiquitination of target proteins that are either negative regulators of DNA repair or are positive regulators of growth proliferation, leading either to their degradation or to an alteration of their activity. The goal of our studies is to characterize the role of *BRCA1*-dependent E3 ubiquitination in the prevention of breast cancer. Our specific aims address the physiological significance of a *BRCA1* E3 activity, the identification of potential *BRCA1* target substrates, and an examination of the significance of a *BRCA1* E3 activity to breast cancer biology. In an attempt to understand the biological significance of *BRCA1*-dependent ubiquitination, we have generated the reagents to reconstitute *BRCA1*-dependent ubiquitination using E1 (Uba1), E2 (UbcH5b), and full-length recombinant *BRCA1* expressed in baculovirus. We are also currently expressing full-length *BARD1* in bacteria and in baculovirus. To determine whether *BRCA1* and *BARD1* truly function as an ubiquitin protein ligase, we will determine whether the full-length *BRCA1* and *BARD1* heterodimer functions to ubiquitinate model bacterial substrate proteins or exhibits auto-ubiquitination activity. It is expected that only full-length *BRCA1* and *BARD1* proteins will confer physiologically relevant substrate specificity. Because of this, these full-length proteins will be used to identify substrates of *BRCA1* using an *in vitro* ubiquitination assay. We are currently testing Estrogen Receptor α , RNA Polymerase II large subunit, and CDC25A as potential *BRCA1/BARD1* substrates. In the event that E1, E2, and recombinant *BRCA1/BARD1* are not sufficient for the ubiquitination of putative substrates *in vitro*, we are also generating nuclear extracts from cell lines deficient in the expression of *BRCA1* to provide proteins that may be required for *BRCA1*-dependent ubiquitination of substrates.

BODY

Task 1: Months 1-6

To determine if wildtype *BRCA1* versus cancer predisposing RING finger mutants of *BRCA1* function as E3 enzymes to ubiquitinate proteins.

- a. To determine if the wildtype full length *BRCA1* protein and a glutathione-S transferase (GST)-*BRCA1* RING finger domain fusion protein function in association with E1 and E2 to ubiquitinate proteins. (Months 1-6)
- b. To determine if cancer predisposing mutations of *BRCA1* in the RING finger domain abrogate the ubiquitinating activity of *BRCA1*. (Months 1-6)

At the time of the submission of this grant, only one publication existed that described the putative E3 activity of a *BRCA1* protein fragment containing the RING finger domain of *BRCA1* (8). However, by the time this grant was awarded and activated, two additional reports were published that further addressed the putative E3 activity of *BRCA1* amino-terminal protein fragments. One report demonstrated the loss of *BRCA1*'s E3 activity and the loss of protection from radiation induced hypersensitivity upon the mutation of the *BRCA1* RING finger domain (9). The other described the importance of a *BRCA1-BARD1* heterodimer for the E3 activity of *BRCA1* and the loss of this E3 activity upon mutation of the *BRCA1* RING finger domain (7). Just recently, an additional publication described the auto-ubiquitination activity of a *BRCA1/BARD1* heterodimer (4). Each of these published reports described the putative E3 activity of *BRCA1* using amino-terminal protein fragments of *BRCA1* and describes either auto-ubiquitination or the ubiquitination of model substrates. No report has yet studied the ubiquitination activity of a full-length heterodimer of *BRCA1* and *BARD1* which is the protein complex most likely to confer physiologically relevant substrate specificity.

Our efforts during the past year have focused on developing the reagents required to study the E2-dependent ubiquitination activity of full-length recombinant BRCA1 and BARD1. In order to set up an *in vitro* BRCA1-dependent ubiquitination system, we have generated plasmids to express GST- and 6xHis-tagged ubiquitin containing the phosphorylation site for Protein kinase A (PKA). We have expressed and purified these proteins to near homogeneity (**Fig. 1** of Appendix). The PKA site allows the *in vitro* ³²P-labeling of ubiquitin to aid in the visualization of ubiquitinated substrate proteins. We are also generating a Hemagglutinin (HA) tagged ubiquitin to allow the visualization of ubiquitin conjugates using a non-radioactive, western-based chemiluminescent detection system. From our studies, we found that it was not very useful to use an anti-GST antibody or an antibody against the MRGS-6xHis tag to detect BRCA1-dependent ubiquitin-conjugates using a western based method. This is because the GST and MRGS-6xHis antibodies work poorly for western blotting, producing a low signal to noise ratio. To circumvent this problem, we are generating the HA-tagged ubiquitin. The monoclonal antibody against the HA tag (12CA5) is readily available commercially and exhibits little background with high sensitivity. We also have generated several mammalian expression vectors expressing HA- and Myc-tagged ubiquitin for the detection of ubiquitinated species in transfected mammalian cells.

We have also engineered a plasmid to express 6xHis-tagged E1 protein after many unsatisfactory attempts at purifying a non-tagged E1 from the pET vector system. The non-tagged E1 in the pET vector was expressed only at low levels in soluble form and was difficult to purify. The 6xHis-tagged E1 we have expressed is soluble and can be purified to approximately 70% purity over one column (**Fig. 2** of Appendix). We have also purchased E1 from Boston Biochem for experiments that require non-tagged E1 protein. We have also generated an expression plasmid to express the E2, Ubch5b, which has been shown to support BRCA1-dependent ubiquitination (8). Ubch5b has been expressed and purified to homogeneity (**Fig. 2** of Appendix). Both 6xHis-tagged E1 and E2 were cloned using Polymerase Chain Reaction (PCR) cloning and were confirmed by DNA sequencing. Using the recombinantly expressed 6xHis-ubiquitin, E1, and 6xHis-tagged Ubch5b, we performed a charging reaction to determine whether our reagents were able to form a high energy bond thiolester on the conserved active site cysteine of Ubch5b. Our results indicate that our reagents are able to efficiently charge Ubch5b (**Fig. 4** of Appendix).

GST-tagged BRCA1 wildtype (WT) and RING finger mutant C64G were expressed from plasmids obtained from collaborator Dr. Wen-Hwa Lee. Both BRCA1 proteins are expressed as amino-terminally tagged GST proteins fused to the first 302 amino acids of BRCA1 containing the RING finger domain. Both have been expressed and purified to approximately 80-90% homogeneity (**Fig. 3** of Appendix). These proteins have been used in ubiquitination assays, but BRCA1-dependent ubiquitination of bacterial proteins has been difficult to observe over the background in our assay. After consultation with Dr. Richard Baer, it is now apparent to us that unless BARD1 is included in the ubiquitination assay, the BRCA1 ubiquitinated species are often difficult to visualize. Dr. Baer has sent us BARD1 constructs which we are now using to express epitope tagged full-length BARD1 in bacteria and baculovirus. These constructs are currently in progress. Through a collaboration with Drs. Thomas Boyer and Wen-Hwa Lee, we have expressed full-length BRCA1 in baculovirus-infected Sf9 cells. This BRCA1 is carboxyl-terminally tagged with the HA epitope to allow the purification of non-denatured BRCA1 from 12CA5 beads using the HA peptide. Although this strategy works well to purify small amounts of relatively pure BRCA1, it is very difficult to elute moderate quantities of BRCA1 from the 12CA5 beads. This is most likely due to the BRCA1 C-terminal (BRCT) repeats located at the carboxyl-terminus of BRCA1 which recent crystal structures have indicated are folded back on itself making this region less accessible (10). Because of this difficulty, we are currently generating recombinant baculovirus for the expression of amino-terminally FLAG-tagged BRCA1. Until we obtain FLAG-tagged BRCA1, we are using baculovirus infected cell lysates to determine whether full-length WT BRCA1 can support the ubiquitination of model substrates or the auto-ubiquitination of BRCA1/BARD1. These studies are currently in progress. We are also trying to express the full-length C64G RING finger mutant of BRCA1 in baculovirus. Unfortunately, the HA-tagged C64G BRCA1 mutant is expressed in baculovirus-infected Sf9 cells at a level ten fold lower than the WT BRCA1 protein. We are currently working on methods to increase the expression of the C64G BRCA1 mutant. Thus far, the expression of full-length BRCA1 and BARD1 has been a limiting factor in our completion of Task 1. BRCA1 is a very large protein and has been difficult for many researchers to express in moderate quantities for biochemical assays. However, we feel that with the studies we have performed thus far, we will move beyond the technical difficulties and accomplish this task in the next 3 months.

Task 2: Months 1-30

To identify the key physiological target (substrate) or targets of BRCA1 ubiquitination.

- a. To study predicted targets of BRCA1 for BRCA1-dependent ubiquitination. Predicted targets of BRCA1 ubiquitination include BRCA1 interacting proteins and putative functional targets of BRCA1 such as the Estrogen Receptor α and RNA Polymerase II large subunit. (Months 6-12)

We have generated expression plasmids for human CDC25A, RNA Polymerase II large subunit, and Estrogen Receptor α . In a preliminary ubiquitination experiment using the amino terminus of BRCA1 (1-302) fused to GST and ^{35}S -labeled CDC25A, we were unable to detect ubiquitination of CDC25A. Further in vitro ubiquitination assays are awaiting the expression and purification of full-length BRCA1 and BARD1 which we hope to complete in the next 3 months. In the event that the baculovirus expressed BRCA1 and BARD1 is not sufficient to support the ubiquitination of bona fide BRCA1 substrates, it may be necessary to provide the BRCA1 E3 from mammalian cells which have been subjected to DNA damaging agents. To aid in these efforts, we are also generating stable cell lines that express epitope-tagged full-length BRCA1, BRCA1 C64G RING finger mutant, and BARD1. These stable cell lines will be used to purify the BRCA1/BARD1 E3 complex from DNA damaged or undamaged mammalian cells by virtue of the epitope tag. This potentially more physiological BRCA1/BARD1 E3 complex will then be used for ubiquitination assays. Using a stable cell line expressing the BRCA1 RING finger mutant C64G may also aid in the identification of BRCA1 substrates in vivo. It is predicted that we may be able to co-precipitate a putative BRCA1 substrate that is expected to associate more tightly with the BRCA1 RING finger mutant in cells. Because stable cell lines expressing epitope tagged BRCA1 can be treated to various environmental stresses prior to the purification of the BRCA1/BARD1 E3 complex, this complex may be more useful for the identification of bona fide BRCA1 substrates.

- b. To identify BRCA1 ubiquitinated proteins using a small pool screening approach (In Vitro Expression Cloning or IVEC) and nuclear extracts derived from BRCA1 +/+ and BRCA1 -/- cell lines. (Months 1-12 = Generation of mouse library; Months 12-30 = Screening of library)

We have begun to generate a library from BG-1 ovarian adenocarcinoma cells deficient in BRCA1 protein expression. These cells are estrogen receptor positive and constitutively express anti-sense RNA to BRCA1. These cells were provided by collaborator Dr. Lois A. Annab. We have chosen to use BG-1 cells for our library since they are responsive to estrogen which may be an important factor for the proper expression and regulation of the BRCA1 E3 activity. The BRCA1 mouse embryonic fibroblast cells are estrogen receptor negative and they are also embryonic cells. Although we may still utilize these cells for the generation of a library for in vitro expression cloning, these cells may not express the normal BRCA1 E3 activity and thus, may not be optimal for the search for BRCA1 substrates.

Task 3: Months 24-36

To determine the significance of BRCA1 E3 ubiquitinating activity in cancer cell biology.

- a. To determine if the ubiquitinating activity of wildtype BRCA1 versus cancer predisposing mutations of BRCA1 is regulated upon DNA damage. (Months 24-36)
- b. To study the E3 activity of BRCA1 in different cancer cell lines. (Months 24-36)

We will begin these studies in year three of this award.

KEY RESEARCH ACCOMPLISHMENTS

- Development of an in vitro ubiquitination system to study the ubiquitin ligase activity of the RING finger domain of BRCA1 (E1, E2-Ubch5b, GST-BRCA1(1-302), GST-BRCA1(1-302, C61G/C64G RING finger mutant)
- Expression of full-length C-terminally HA-tagged BRCA1 from baculovirus (in collaboration with Thomas G. Boyer)

REPORTABLE OUTCOMES

Abstracts:

Michelle Bromhal, Wei Tan, Thomas G. Boyer, and P. Renee Yew. 2002. The Role of BRCA1-Dependent Ubiquitination in Breast Cancer. The First International Conference: Ubiquitin, Ubiquitin-like Proteins, and Cancer. The University of Texas M.D. Anderson Cancer Center, Houston, Texas.

Manuscripts:

None.

Awards:

Yew, P. Renee
Career Development Award DAMD17-02-1-0589 (2002-2006)

CONCLUSIONS

We have generated many reagents (tagged ubiquitin, E1, E2-UbcH5b, GST-BRCA1 (1-302) WT and C64G) which are critical for our studies of the putative BRCA1 ubiquitin protein ligase activity. In the past year, it has become apparent that a key to understanding the activity of BRCA1 in the ubiquitination of proteins is the inclusion of BARD1 in BRCA1 ubiquitination studies. We are now focusing our efforts on expressing full-length BRCA1 and BARD1 proteins for the study of putative BRCA1 substrates. Our studies address a highly novel activity of BRCA1 that could have a large impact on our understanding of familial breast cancer development. It is our hope that these studies will help to understand the putative E3 activity of BRCA1 in the cell and help to identify important targets of the BRCA1 E3 activity. This discovery could not only alter the manner in which all future studies are directed on the function of BRCA1 in the cell, but could also open many new avenues for the development and implementation of drugs for the treatment of breast cancer.

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APPENDICES

Appendix 1: Figures containing data as referenced within the “Body” of the Annual Report.

Appendix 2: Current curriculum vitae.

Figure 1

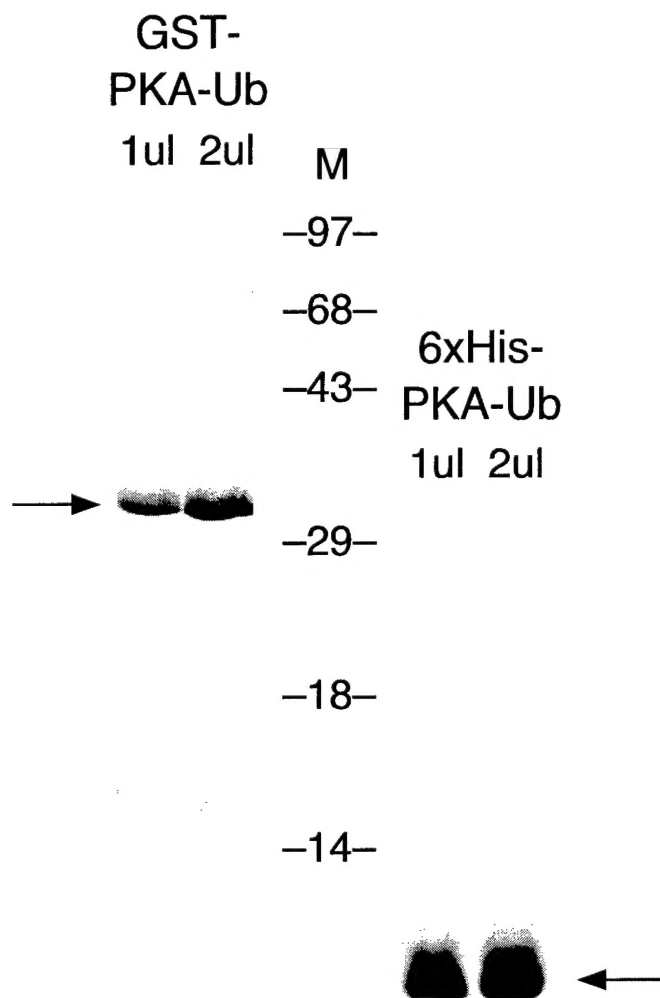


Figure 1: Purified Recombinant Ubiquitin. Coomassie blue stained gel showing bacterially expressed and purified glutathione-S-transferase (GST) tagged ubiquitin (Ub) with protein kinase A (PKA) phosphorylation site (left) and hexa-histidine (6xHis) tagged ubiquitin (Ub) with PKA site (right). Protein bands are indicated by arrows. Molecular weight markers (M) are indicated in kilodalton.

Figure 2

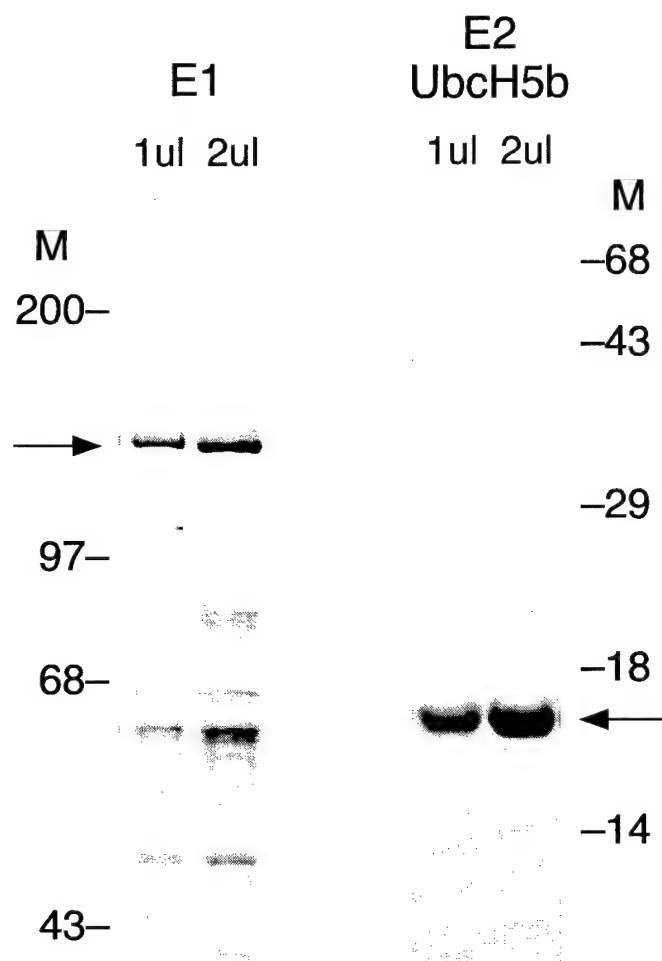


Figure 2: Purified recombinant E1 and E2. Coomassie blue stained gel showing bacterially expressed and purified hexa-histidine (6xHis) tagged E1 (Uba1) (left) and E2 (UbcH5b) (right). Protein bands are indicated by arrows. Molecular weight markers (M) are indicated in kilodalton.

Figure 3

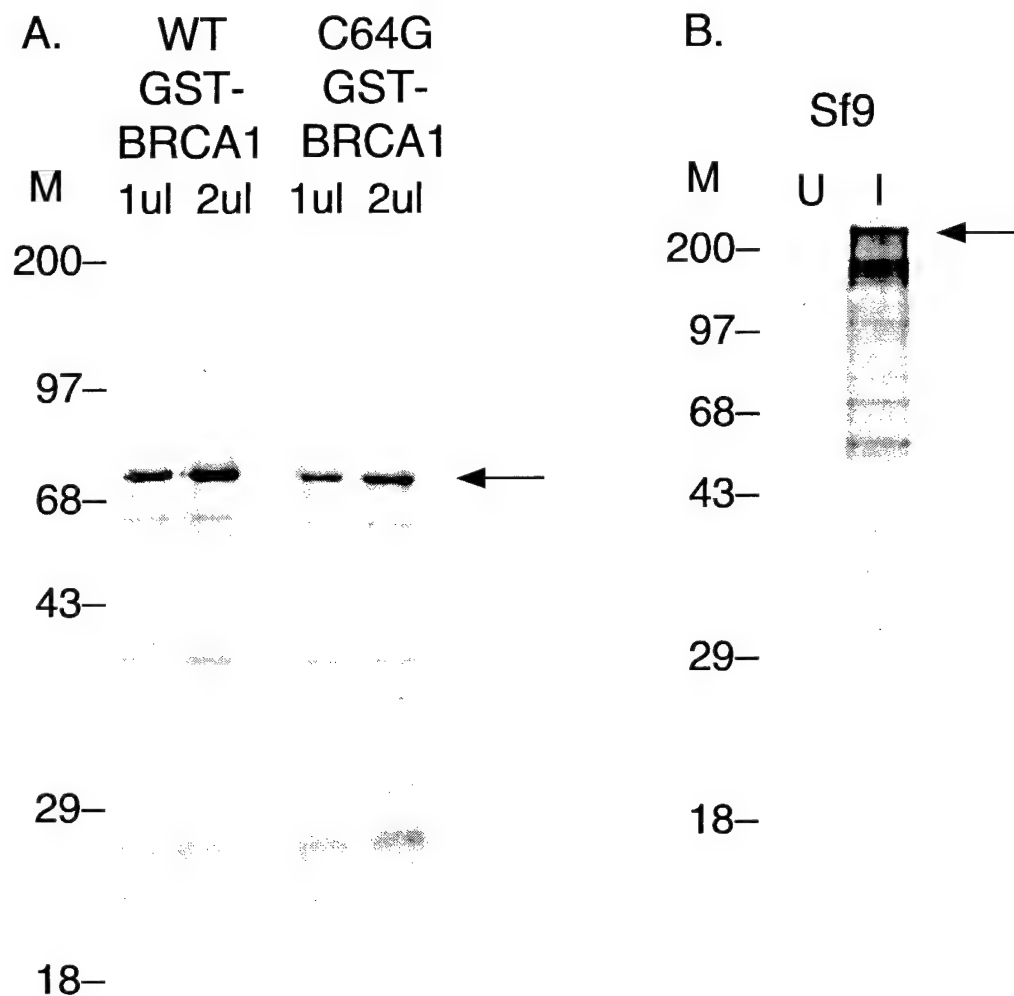


Figure 3: Recombinant BRCA1. **A.** Coomassie blue stained gel showing bacterially expressed and purified GST-tagged wildtype (WT) BRCA1(1-302) (left) and GST-tagged mutant C64G BRCA1(1-302) (right). **B.** Immunoblot of C-terminally HA-tagged BRCA1 from baculovirus infected (I) and uninfected (U) Sf9 cells using BRCA1 antibody 6B4. Protein bands are indicated by arrows and molecular weight markers (M) are indicated in kilodalton.

Figure 4

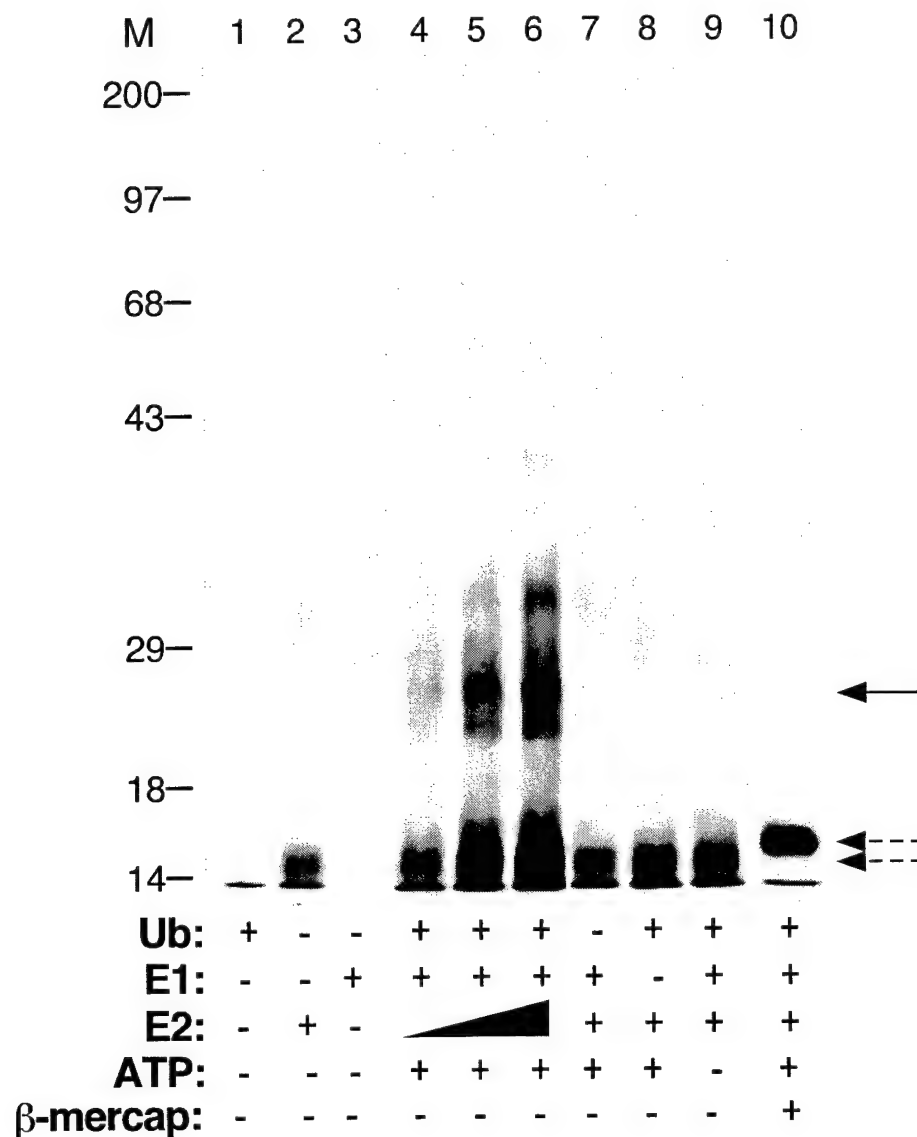


Figure 4: E2 Thiolester Formation Assay. Immunoblot using MRGS-6xHis antibody, 6xHis-Ubiquitin (Ub), E1 (Boston Biochem, no tag), E2 (6xHis-UbcH5b) at increasing concentrations (125ng, 250ng, 500ng), and ATP. Ubiquitin was used at 250ng per reaction, E1 was used at 150ng per reaction, and E2 was used as indicated except in lane 2 where 125ng was used. The negative control reaction contains reducing agent, β -mercaptoethanol (β -mercap, lane 10). Ubiquitin-charged E2 band is indicated by the solid arrow, E2 is indicated by the dashed arrows, and molecular weight markers (M) are indicated in kilodalton.

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Education and Research Training

June 1983	A.B. in Genetics University of California, Berkeley, California
June 1985	M.A. in Biochemistry and Molecular Biology University of California, Santa Barbara, California
1985-1987	Research Associate Amgen Inc., Thousand Oaks, California (Advisor: Arlen Thomason, Ph.D.)
December 1993	Ph.D. in Microbiology and Molecular Genetics University of California, Los Angeles, California (Advisor: Arnold J. Berk, M.D.)
1993-1998	Post-doctoral Research Fellow Department of Cell Biology Harvard Medical School, Boston, Massachusetts (Advisor: Marc W. Kirschner, Ph.D.)
November 1998 to present	Assistant Professor Department of Molecular Medicine, Institute of Biotechnology University of Texas Health Science Center, San Antonio, Texas

Teaching Experience

1984-1985	Teaching Assistant and Tutor Department of Biochemistry and Molecular Biology University of California, Santa Barbara, California
1987-1988	Teaching Assistant Department of Microbiology and Molecular Genetics University of California, Los Angeles, California
1999-2002	Instructor, Seminars in Molecular Medicine, Spring Instructor, Advanced Molecular Cell Biology, Fall
2000-2001	Instructor, Methods in Molecular and Cellular Biology, Fall Department of Molecular Medicine University of Texas Health Science Center, San Antonio, Texas

Honors and Awards

1989-1992	NIH Pre-doctoral Trainee, Training Grant in Genetics
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1993	Sidney C. Rittenberg Award University of California, Los Angeles, California
1994-1997	Jane Coffin Childs Memorial Fund Post-doctoral Fellowship, New Haven, Connecticut
1997-2000	The Leukemia and Lymphoma Society Special Fellowship, New York, New York
2002-2006	Department of Defense Breast Cancer Research Program Career Development Award, Fort Detrick, Maryland

Publications

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Manuscripts Submitted

None.

Manuscripts in Preparation

1. Li-Chiou Chuang, Cathie M. Pfleger, Hui-Min Tseng, Karen Block, Carlos Herrera, Marc W. Kirschner, and P. Renee Yew. Identification of the minimal sequences necessary and sufficient for nuclear ubiquitination and degradation of the *Xenopus* cyclin-dependent kinase inhibitor, p27^{Xic1}. *Manuscript in preparation*.
2. Li-Chiou Chuang and P. Renee Yew. PCNA recruits CDK inhibitor, p27^{Xic1}, to sites of initiation and couples its proteolysis to DNA polymerase switching. *Manuscript in preparation*.
3. Karen Block, Joanna Bloom, Michele Pagano, and P. Renee Yew. The acidic tail domain of human CDC34 is required for p27^{Kip1} ubiquitination and complementation of a *cdc34* temperature sensitive strain in budding yeast. *Manuscript in preparation*.

Abstracts

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